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**The effects of heavy metal ions, phthalates and ochratoxin A on oxidation of carcinogenic
aristolochic acid I causing Balkan endemic nephropathy**

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Running headline: AAI oxidation and other factors of BEN

KEYWORDS

aristolochic acid; oxidation; cytochrome P450; heavy metals; phthalates; ochratoxin A

OBJECTIVES: Balkan endemic nephropathy (BEN) is a chronic progressive fibrosis associated with upper urothelial carcinoma (UUC). Aetiology of BEN is still not fully explained. Although carcinogenic aristolochic acid I (AAI) was proven as the major cause of BEN/UUC, this nephropathy is considered to be multifactorial. Hence, we investigated whether other factors considered as potential causes of BEN [a mycotoxin ochratoxin A (OTA), Cd, Pb, Se and As ions and organic compounds (i.e. phthalates) released from lignite deposits in BEN areas] can influence detoxication of AAI, whose concentrations are crucial for BEN development.

METHODS: Oxidation of AAI to 8-hydroxyaristolochic acid I (AAIa) in the presence of Cd, Pb, Se, As ions, dibutylphthalate (DBP), butylbenzylphthalate (BBP), bis(2-ethylhexyl)phthalate (DEHP) and OTA by rat liver microsomes was determined by HPLC.

RESULTS: Only OTA, cadmium and selenium ions, and BBP inhibited AAI oxidation by rat liver microsomes. These compounds also inhibited activities of CYP1A1 and/or CYP2C6/11 catalysing AAI demethylation in rat livers. Therefore, these CYP inhibitions can be responsible for a decrease in AAIa formation. When the combined effects of these compounds were investigated, the most efficient inhibition was caused by OTA combined with BBP and selenium ions.

CONCLUSION: The results show low effects of BBP, cadmium and selenium ions, and/or their combinations on AAI detoxication. No effects were produced by the other metal ions (Pb, As) and phthalates DBP and DEHP. This finding suggests that they do not influence AAI-mediated BEN development. In contrast, OTA might influence this process, by inhibition of AAI detoxication.

ABBREVIATIONS

AA – aristolochic acid

AAI – 8-methoxy-6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid

AAIa – 8-hydroxyaristolochic acid I

AAII – 6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid

AAN – aristolochic acid nephropathy

BBP – butylbenzylphthalate

BEN – Balkan endemic nephropathy

CYP – cytochrome P450

dA-AAI – 7-(deoxyadenosin- N^6 -yl)aristolactam I

DBP – dibutylphthalate

DEHP – bis(2-ethylhexyl)phthalate

dG-AAI – 7-(deoxyguanosin- N^2 -yl) aristolactam I

EROD – 7-ethoxyresorufin-*O*-deethylase

HPLC – high performance liquid chromatography

IARC – International Agency for Research on Cancer

NADP⁺ – nicotinamide adenine dinucleotide phosphate (oxidised)

NADPH – nicotinamide adenine dinucleotide phosphate (reduced)

NQO1 – NAD(P)H:quinone oxidoreductase

OTA – ochratoxin A

Pb(Ac)₂ – lead acetate

r.t. – retention time

TP53 – tumour suppressor gene

UUC – upper urothelial carcinoma

INTRODUCTION

Balkan endemic nephropathy (BEN) is a chronic progressive renal fibrosis affecting rural population in Bulgaria, Bosnia and Herzegovina, Romania, Croatia and Serbia along Danube river basin (Stefanovic, 1983; Radanovic, 2002). The unique feature of this disease is that BEN seems to be a familial, but not inherited occurring in several endemic areas (Toncheva *et al.* 1998; Radanovic, 2002; Grollman, 2013). Moreover, this serious disease is closely associated with upper urothelial carcinomas (UUC) of the renal pelvis and ureter (Stefanovic, 1983; Jankovic *et al.* 1988; Nikolic *et al.* 2002). Although BEN has been studied for more than 50 years, aetiology of this nephropathy is still a

matter of debate. There are several hypotheses suggesting that BEN/UUC is the multifactorial disease which might be caused by environmental compounds such as: (i) aristolochic acid (AA) (Ivic, 1969; Hranjec *et al.* 2005; Arlt *et al.* 2002a, 2007; Grollman *et al.* 2007), (ii) mycotoxins [i.e. ochratoxin A (OTA), citrinine] (Radic *et al.* 1997; Pfohl-Leszkowicz, 2009), (iii) heavy metal ions (Nichifor *et al.* 1985; Long *et al.* 2001; Karmaus *et al.* 2008) and (iv) organic compounds released from lignite deposits in the endemic areas (Feder *et al.* 1991; Tatu *et al.* 1998).

During the last decade, AA was identified as the main cause of this environmental disease (Ivic, 1969; Arlt *et al.* 2002a, 2007; Grollman *et al.* 2007; Stiborova *et al.* 2008). The AA was suggested as possible cause of BEN/UUC for the first time in late 1960s (Ivic, 1969). This plant alkaloid found in *Aristolochia* species was found in wheat used for home-prepared bread (Ivic, 1969; Jelakovic *et al.* 2012; Gokmen *et al.* 2013). The plant extract of AA is a mixture of structurally related nitrophenanthrene carboxylic acids whose major components are aristolochic acid I (AAI) and aristolochic acid II (AAII). AAI is supposed to be the predominant compound responsible for BEN development. AA and herbal products derived from genera *Aristolochia* have been classified by International Agency for Research on Cancer (IARC) as carcinogenic to human (Group 1) (Grosse *et al.* 2009). Furthermore, this nephrotoxic and carcinogenic agent was found to cause also another disease similar to BEN, aristolochic acid nephropathy (AAN) (Cosyns *et al.* 1994; Arlt *et al.* 2002b, 2007; Debelle *et al.* 2008; Schmeiser *et al.* 1996, 2009; Gokmen, 2013).

In contrast to the finding that AAI might directly cause interstitial nephropathy, metabolic activation of this alkaloid to species forming DNA adducts is a necessary step for AAI-induced malignant transformation (Cosyns *et al.* 1994; Arlt *et al.* 2002b; Schmeiser *et al.* 1996, 2009; Chen *et al.* 2012). In organisms, AAI can be either reductively activated to *N*-acylnitrenium ion leading to AAI-DNA adduct formation or oxidatively detoxified to an *O*-demethylated product, 8-hydroxyaristolochic acid (aristolochic acid Ia, AAIIa; Figure 1) (Arlt *et al.* 2002b, 2007; Grollman *et al.* 2007; Stiborova *et al.* 2014a, 2014b). The AA-DNA adducts formed from activated AAI with adenosine and guanosine residues in DNA were found in BEN and AAN patients (Schmeiser *et al.* 1996; Bieler *et al.* 1997; Arlt

et al. 2002a). The most persistent DNA adduct, 7-(deoxyadenosin- N^6 -yl)-aristolactam I (dA-AAI), is proposed to cause a characteristic AT→TA transversion mutations which have been detected in the *TP53* tumour suppressor gene in tissues of patients from the endemic areas. Such AT→TA transversions are responsible for tumour development in patients suffering from BEN and AAN (Arlt *et al.* 2007; Grollman *et al.* 2007; Stiborova *et al.* 2008; Hollstein *et al.* 2013). These findings indicate that the concentration of AAI in organisms is essential for both renal injury and induction of UUC initiated by activated AAI. The effective concentration of AAI in organism is dictated by its metabolism. Since AAI can be both bio-activated to reactive species forming AAI-DNA adducts resulting in cancer development and detoxified to AAIa, these reactions might significantly modulate the AAI toxic/genotoxic potential (Stiborova *et al.* 2008, 2011a, 2012, 2013b; Arlt *et al.* 2011).

A common feature of BEN/UUC is that not all individuals exposed to AAI suffer from these diseases (Arlt *et al.* 2002b, 2011; Stiborova *et al.* 2008, 2012, 2013b, 2015; Jelakovic *et al.* 2012). This phenomenon might be explained by different efficiencies of enzymes participating in metabolism of AAI and by genetic sensitivity of individuals (Stiborova *et al.* 2001, 2003, 2008, 2013b; Toncheva *et al.* 2004; Grollman, 2013). Therefore, detailed understanding of enzymes involved in AAI metabolism (activation and/or reduction) is crucial for risk assessment of AA exposure.

The major enzymes involved in reductive bio-activation of AAI are NAD(P)H:quinone oxidoreductase (NQO1) and cytochromes P450 (CYP) of the 1A subfamily in humans and mouse or rat animal models (Arlt *et al.* 2011; Stiborova *et al.* 2008, 2011a, 2012, 2013b, 2014a, 2014b; Barta *et al.* 2014). The most efficient enzymes participating in AAI detoxication are human, mouse and rat CYP1A1/2 and rat and human CYP2C (Arlt *et al.* 2011; Stiborova *et al.* 2011b, 2012, 2013b).

However, it is still not exactly known whether the other compounds suggested to be responsible for BEN development might influence the AAI-induced BEN. Hence, in this work, we investigated whether OTA, heavy metal ions and organic chemicals released from lignite deposits in the endemic areas can influence the detoxication of AAI, whose concentrations are crucial for BEN development. Heavy metal ions and organic compounds were selected according to data from epidemiologic

studies (Radic *et al.* 1997; Karmaus *et al.* 2008; Pfohl-Leszkowicz, 2009; Yordanova *et al.* 2010; Maharaj *et al.* 2014).

MATERIALS AND METHODS

Chemicals.

AAI sodium salt, CdCl_2 , $\text{Pb}(\text{CH}_3\text{COO})_2$, Na_2SeO_3 , $\text{Na}_2\text{HASO}_4 \cdot 7\text{H}_2\text{O}$, dibutylphthalate (DBP), butylbenzylphthalate (BBP) and bis(2-ethylhexyl)phthalate (DEHP) as well as other chemicals were purchased from Sigma Chemical Co. (St. Louis MO, USA). All chemical were of 97% purity or better.

Preparation of rat hepatic microsomal fraction.

Microsomes were prepared from liver of untreated Wistar rats by differential centrifugation as described previously (Indra *et al.* 2014; Stiborova *et al.* 2013a).

AAIa formation in the presence of heavy metal ions/phthalates/OTA.

Incubation mixtures, in a final volume 500 μL , consisted of 100 mmol.L^{-1} potassium buffer (pH 7.4), NADPH-generation system (1 mmol.L^{-1} NADP^+ , 10 mmol.L^{-1} $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1 U/mL glucose-6-phosphate dehydrogenase), 1–100 $\mu\text{mol.L}^{-1}$ of heavy metal ions/phthalates/OTA, 0.25 mg rat hepatic microsomes and 10 $\mu\text{mol.L}^{-1}$ AAI. CdCl_2 , $\text{Pb}(\text{CH}_3\text{COO})_2$, Na_2SeO_3 , $\text{Na}_2\text{HASO}_4 \cdot 7\text{H}_2\text{O}$ were dissolved in distilled water and OTA was dissolved in 0.1 mol.L^{-1} NaHCO_3 (pH 7) whereas phthalates were prepared in acetonitrile. Incubations with microsomes were carried out at 37 °C for 10 min and AAI oxidation to AAIa was linear up to 25 min (Levova *et al.* 2011; Stiborova *et al.* 2012). Control incubations were carried out (i) without microsomes, (ii) without NADPH-generating system or (iii) without AAI. AAI and AAIa were analysed by high performance liquid chromatography (HPLC) as described (Sistkova *et al.* 2008; Levova *et al.* 2011; Stiborova *et al.* 2012).

HPLC analysis of AAIa formation.

AAI and its *O*-demethylated metabolite (AAIa) were extracted from incubations with ethyl acetate (2 \times 1 mL), the extracts were evaporated to dryness and the residues redissolved in 30 μL of methanol and subjected to reverse-phase HPLC. HPLC was performed with a reversed phase column (Nucleosil

100-5 C₁₈, 25 × 4.0 mm, 5 mm; Macherey-Nagel) preceded by a C-18 guard column, using a linear gradient of acetonitrile (20–60% acetonitrile in 55 min) in 100 mmol.L⁻¹ triethylammonium acetate with a flow rate of 0.5 mL.min⁻¹. A Dionex HPLC pump P580 with UV/VIS UVD 170S/340S spectrophotometer detector was set at 250 nm and CHROMELEON™ 6.01 integrator was used for integration of peaks. AAla and AAI eluted with retention times (r.t.) of 24.5 and 37.7 min, respectively. The product eluting at 24.5 min was identified as AAla by mass spectrometry previously (Sistkova *et al.* 2008; Levova *et al.* 2011; Stiborova *et al.* 2011b).

Determination of CYP1A1/2 and CYP2C6/11 enzyme activities.

In rat hepatic microsomes, CYP1A1/2 was determined by ethoxyresorufine-*O*-deethylation (EROD) (Burke *et al.* 1994). Enzyme activity of CYP1A1 was measured as capability of Sudan I oxidising (Stiborova *et al.* 2002). CYP2C6/11 activities in rat microsomes were characterised as well: CYP2C6 was measured with diclofenac as a marker substrate (Kaphalia *et al.* 2006) and CYP2C11 activity was determined as testosterone 16 α -hydroxylation (Yamazaki *et al.* 2006). The effect of heavy metal ions/phthalates/OTA on the above mentioned enzyme activities was carried out by addition of compounds tested to incubation mixtures in a final concentration of 100 μ mol.L⁻¹.

Statistical analyses.

For statistical data analysis we used Student's *t*-test. All *P*-values are two-tailed and considered significant at the 0.001 level.

RESULTS AND DISCUSSION

Oxidation of AAI to AAla in the presence of the heavy metal ions, phthalates and ochratoxin A.

In rat microsomes, AAI was oxidised to one metabolite eluted by HPLC at r.t. of 24.5 minutes (Figure 2). This metabolite was previously identified by positive MALDI-TOF-TOF analysis as AAla (Levova *et al.* 2011; Stiborova *et al.* 2011b). Because of a low toxicity, AAla was considered to be the detoxication metabolite of AAI (Shibutani *et al.* 2010). The effects of compounds, which were suggested that might contribute to development of BEN/UUC, namely the heavy metal ions,

phthalates and OTA, on detoxication of AAI to AAla catalysed by rat liver microsomes are shown in Figures 3–5. Cadmium and selenium ions inhibited AAla formation (Figures 3A,C) whereas no such effect was found in the presence of Pb^{2+} and arsenate ions (Figures 3B,D). However, the 10-times higher concentration of cadmium and selenium ions than the concentration of AAI was necessary for the significant decrease in AAI oxidation. Of phthalates examined, only butylbenzylphthalate (BBP) inhibited oxidation of AAI to AAla (Figure 4B). The other two studied phthalates (DBP and DEHP) that are known as important toxic environmental pollutants (Ferguson *et al.* 2014; Yan *et al.* 2015) were without this effect (Figures 4A,C). In the case of OTA, the significant inhibition of AAI demethylation by this mycotoxin was found. The 10 and 100 $\mu\text{mol.L}^{-1}$ OTA led to a 23% and 42% decrease in AAla formation, respectively (Figure 5). These findings are consistent with the results found in our former study which demonstrated that OTA is capable of inhibiting AAI oxidation to AAla *in vivo* (Stiborova *et al.* 2015).

Because human population living in the endemic areas might be exposed not only to each of these compounds individually but also to their combination, in the next step of this study we investigated a combined effect of the substances that inhibited AAI oxidation, namely, cadmium and selenium ions, BBP and OTA (Figure 6). Interestingly, although oxidation of AAI to AAla was not influenced by a combination of BBP and OTA, all four contaminants (cadmium and selenium ions, BBP and OTA) added to incubation mixtures led to the significant inhibition of AAla formation, by 34% (Figure 6B). In addition, the most efficient inhibition of AAI demethylation was caused by OTA combined with BBP and selenium ions, by 37% (Figure 6B). Using the other combinations, no additive effects of these combinations compared to inhibition caused by individual compounds were found. This phenomenon is now difficult to be explained. One can speculate that compounds might compete against each other, thereby decreasing the inhibition of enzymes involved in AAI oxidation or intermolecular interactions between the tested compounds might decrease their actual concentrations. These suggestions need, however, to be explored in further studies.

Effect of the heavy metal ions, butylbenzylphthalate and ochratoxin A on enzymatic activities of cytochromes P450.

In order to evaluate the mechanisms of inhibition of AAI oxidation in rat microsomes, the effects of the compounds found to inhibit this reaction (Figures 3–5) on activities of the major enzymes participating in AAI detoxication were analysed. Namely, the effects of cadmium and selenium ions, BBP and OTA on activities of CYP1A and 2C6/11 enzymes were tested. The enzyme activities were determined utilising a marker substrates (see the Material and Methods section). The data shown in Table 1 demonstrate that CYP1A1 activity was inhibited mainly by Cd^{2+} and BBP; a 54 and 75% decrease in a CYP1A1 marker activity (Sudan I oxidation) was found, respectively, whereas the other tested compounds did not influence CYP1A activity. In the case of CYP2C, BBP significantly inhibited activity of CYP2C6, whereas OTA decreased activity of CYP2C11, the CYP enzyme that is predominantly expressed in rat liver (Zacharová *et al.* 2012). Based on these results, the decreased oxidation of AAI to AAIa caused by tested contaminants might be caused by decreased enzyme activities of these CYPs.

Several studies suggested that BEN and AAN may be the same diseases which differ slightly (Grollman *et al.* 2009). One of the differences between BEN and AAN is that whereas BEN is characterised by an insidious onset and slow gradual progression (Grollman *et al.* 2007; Stiborova *et al.* 2008; Jelakovic *et al.* 2012.), AAN is defined as rapidly progressive interstitial renal fibrosis (Vanherweghem *et al.* 1993). One of the reasons responsible for this difference seems to be a different exposure schedule of patients; chronic exposure to low concentrations of AA of population living in BEN areas, whereas exposure to high doses of individuals in which AAN was developed (Gokmen *et al.* 2013). However, based on the results found in the present and our former (Stiborova *et al.* 2015) studies, this difference might also follow from inhibitions of AAI detoxication by the compounds such as heavy metal ions (cadmium and selenium), phthalates (BBP) and OTA, the substances to which BEN/UUC patients are exposed. Although BBP and cadmium and selenium ions were shown to be present in water and lignite samples in the BEN areas (Karmaus *et al.* 2008;

Maharaj *et al.* 2014), there is still not enough information on their exact concentrations in these samples. Therefore, we cannot evaluate whether their concentrations found in this study to inhibit AAI detoxication are valid for the BEN development. Nevertheless, even though there is a study which has demonstrated that a role of heavy metal ions in BEN/UUC development is negligible (Karmaus *et al.* 2008), because of inhibition of AAI detoxication by cadmium and selenium ions found in this work, the participation of the chronic exposure to these ions in the AAI-mediated BEN development cannot be excluded. Chronic OTA intake is common in many populations ranging up to 25 ng.kg⁻¹ body weight and day, and OTA is detectable in plasma in BEN patients at levels up to 3.9 ng.mL⁻¹ (Yordanova *et al.* 2010) but also in healthy controls. The doses to which humans are exposed are lower than the OTA concentrations found in this study to inhibit AAI detoxication. However, drug-drug interactions between AAI and OTA at lower but chronic and life-long doses of human exposure in BEN areas may be different. Therefore, OTA, because of its potency to increase actual concentrations of AAI by inhibition of its detoxication, may enhance the development of AAI-induced UUC in BEN patients.

CONCLUSIONS

The results found in this study demonstrate that AAI detoxication to AAIa is inhibited by OTA, phthalate BBP and partially also by cadmium and selenium ions. Such inhibition is caused by inhibition of the CYP1A1 and 2C enzymes that catalyse this reaction. Even though these inhibitions are not fatal, their contributions to the AAI-mediated development of BEN/UUC, considering mainly the chronic exposure of population living in the endemic areas to these pollutants, cannot be ruled out.

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Tab. 1. The effects of cadmium and selenium ions, BBP, and OTA on enzyme activity of rat CYP1A and 2C.

Pollutant	EROD activity (CYP1A) ^a	Sudan I oxidation (CYP1A1)	Diclofenac 4'-hydroxylation (CYP2C6)	Testosterone 16 α -hydroxylation (CYP2C11)
CdCl ₂	83±0.09*	46±4.27***	89±0.44*	100±6.43
Na ₂ SeO ₃	95±0.83	100±2.62	94±5.59	82±1.01**
OTA	100±7.50	99±4.93	NE ^b	18±1.24***
BBP	83±1.24*	25±1.27***	7±0.26***	68±12.48**

Data are expressed as % of control without pollutants. Values in the table are averages \pm standard deviations of three experiments ($n = 3$). The incubation mixtures contained 0.5 mg.mL⁻¹ microsomal protein, 10 μ mol.L⁻¹ AAI (dissolved in distilled water), the NADPH-generating system containing 1 mmol.L⁻¹ NADP⁺, and 100 μ mol.L⁻¹ pollutant [heavy metal ions dissolved in distilled water, OTA dissolved in 0.1 mol.L⁻¹ NaHCO₃ (pH 7) or BBP dissolved in acetonitrile] (see Materials and Methods). Values significantly different from control incubations without pollutants; *P<0.1, **P<0.01, ***P<0.001 (Student's t-test).

^aIsoforms of CYP whose enzyme activity is measured are shown in brackets.

^bNE, no effect.

Fig. 1. Scheme of detoxication and bio-activation of AAI in organisms. dA-AAI, 7-(deoxyadenosin- N^6 -yl)aristolactam I; dG-AAI, 7-(deoxyguanosin- N^2 -yl)aristolactam I; UGT, UDP glucuronosyl transferase; SULT, sulfotransferase.

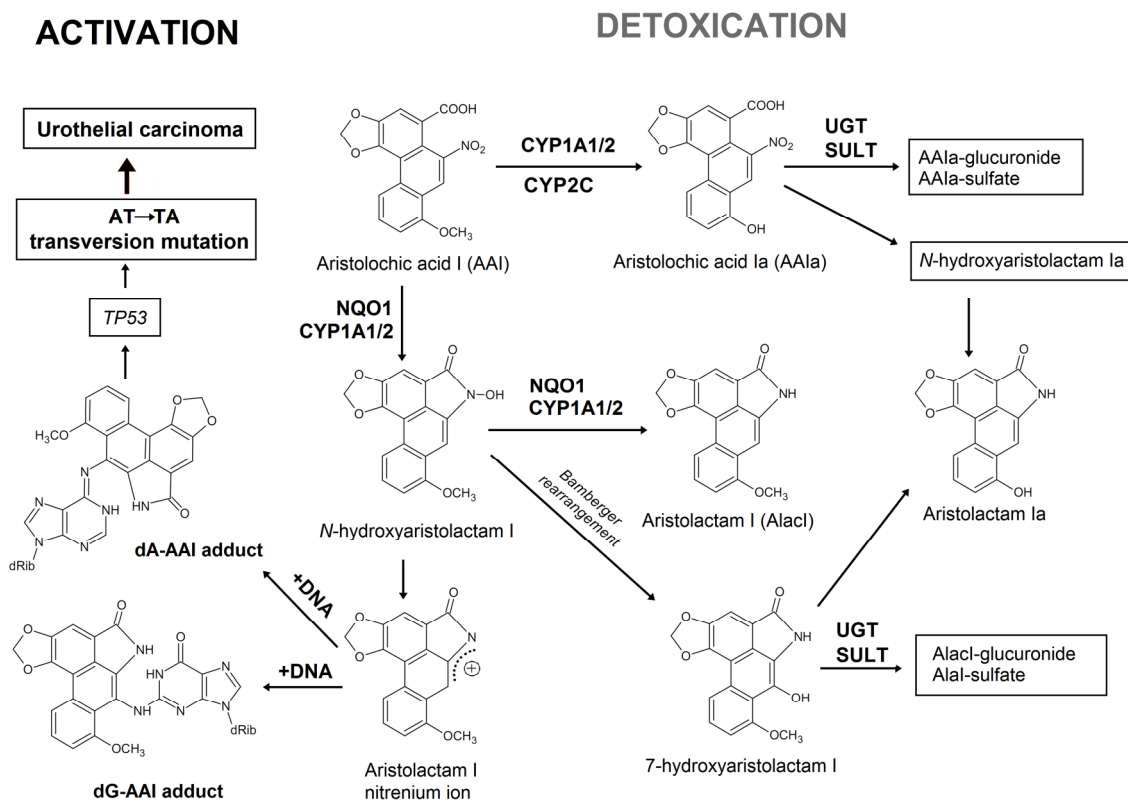


Fig. 2. HPLC of AAI (peak r.t. at 37.7 min) and AAIa metabolite (peak r.t. at 24.5 min) produced by hepatic microsomes of control (untreated) rats incubated with AAI and the NADPH-generating system. The peaks with the characterised metabolite AAIa and the parent AAI are indicated in the chromatograms.

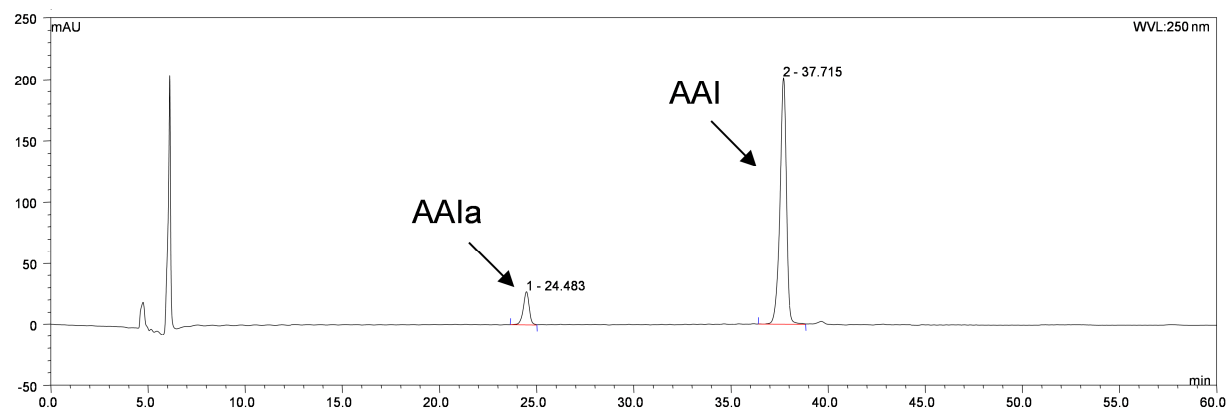


Fig. 3. AAI oxidation to AAla catalysed by rat hepatic microsomes in the presence of heavy metal ions present in incubation mixtures, CdCl_2 (A), $\text{Pb}(\text{CH}_3\text{COO})_2$ [$\text{Pb}(\text{Ac})_2$; B], Na_2SeO_3 (C), $\text{Na}_2\text{HASO}_4 \cdot 7\text{H}_2\text{O}$ (D). Values are given as means \pm standard deviations of three experiments ($n = 3$). Values significantly different from incubation with buffer only: * $P < 0.1$, ** $P < 0.01$ (Student's t-test). The incubation mixtures contained 0.5 mg.mL^{-1} microsomal protein, $1\text{--}100 \text{ }\mu\text{mol.L}^{-1}$ heavy metal ions dissolved in distilled water, the NADPH-generating system containing 1 mmol.L^{-1} NADP^+ , and $10 \text{ }\mu\text{mol.L}^{-1}$ AAI dissolved in distilled water (see Materials and Methods). Numbers above the columns ("F") indicate fold changes in amounts of AAla compared to incubations without heavy metal ions.

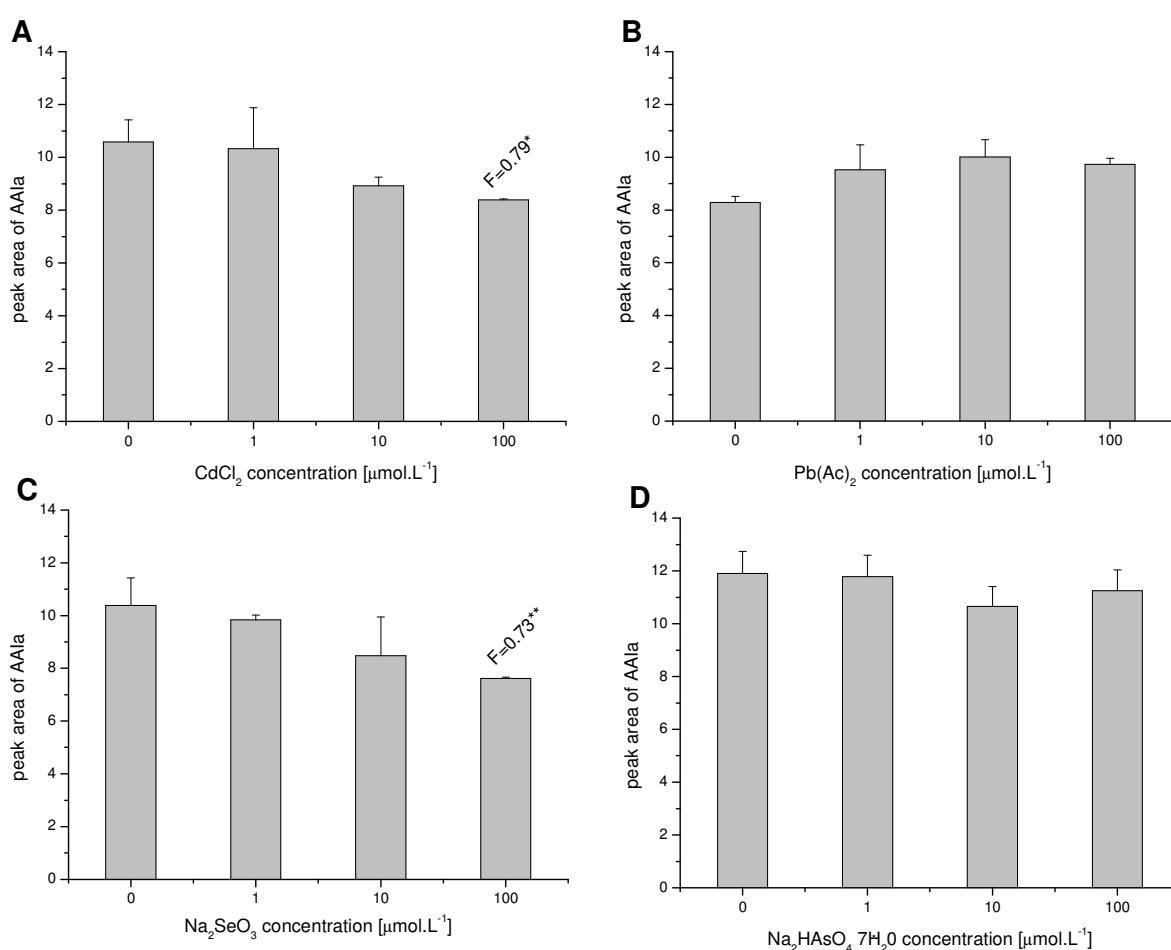


Fig. 4. AAI oxidation to AAla catalysed by rat hepatic microsomes in the presence of phthalates. In incubation mixtures, dibutylphthalate (DBP, A), butylbenzylphthalate (BBP, B) and bis(2-ethylhexyl)phthalate (DEHP, C) all dissolved in acetonitrile were used. Values are given as means \pm standard deviations of three experiments ($n = 3$). Values significantly different from incubation with acetonitrile only: * $P < 0.1$, ** $P < 0.01$ (Student's t -test). The incubation mixtures contained 0.5 mg.mL^{-1} microsomal protein, $1\text{--}100 \text{ }\mu\text{mol.L}^{-1}$ phthalates, the NADPH-generating system containing 1 mmol.L^{-1} NADP^+ , and $10 \text{ }\mu\text{mol.L}^{-1}$ AAI dissolved in distilled water (see Materials and Methods). Numbers above the columns ("F") indicate fold changes in amounts of AAla compared to incubations without phthalates.

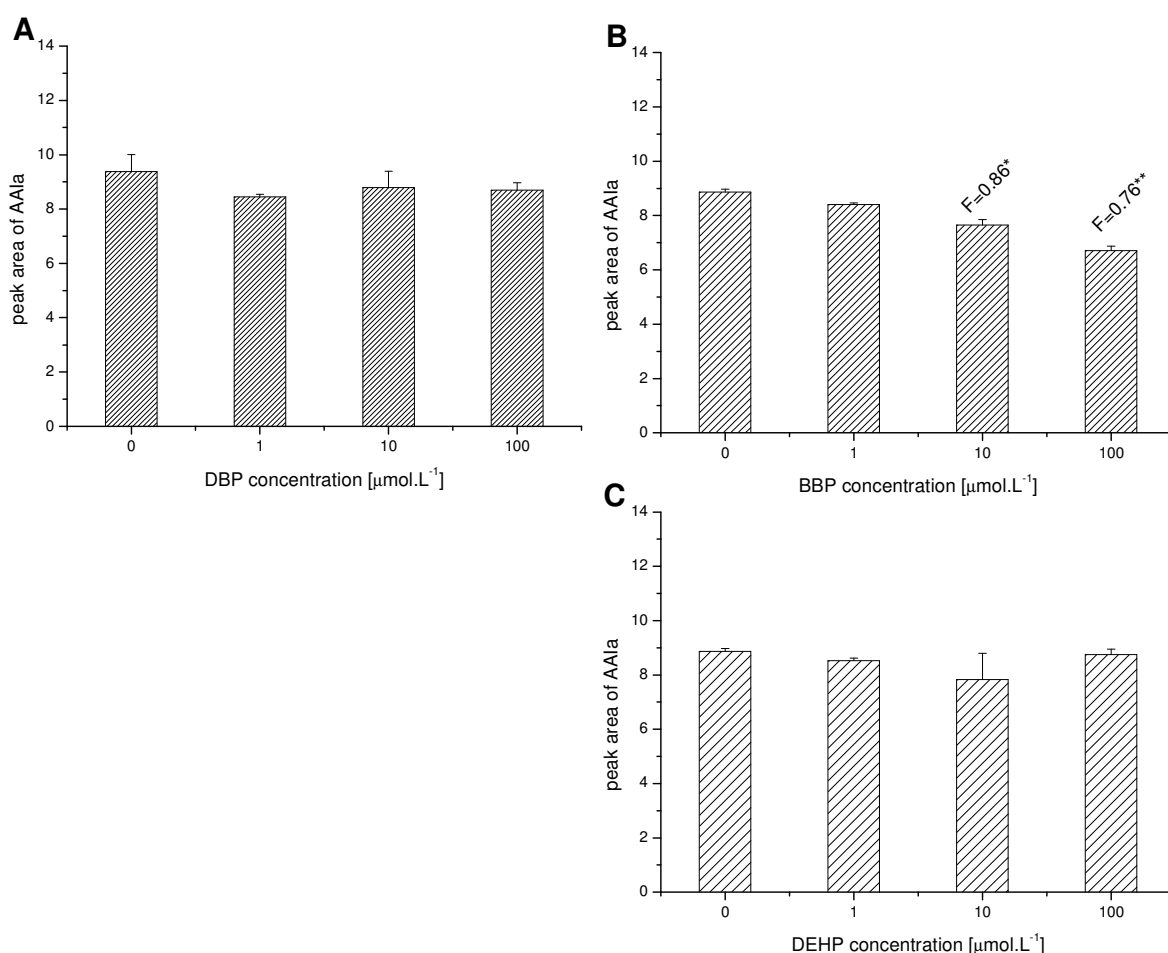


Fig. 5. AAI oxidation to AAla catalysed by rat hepatic microsomes in the presence of OTA dissolved in 0.1 mol.L⁻¹ NaHCO₃ (pH 7). Values are given as means \pm standard deviations of three experiments (n = 3). Values significantly different from incubation with 0.1 mol.L⁻¹ NaHCO₃ (pH 7) only: ***P<0.001 (Student's t-test). The incubation mixtures contained 0.5 mg.mL⁻¹ microsomal protein, 1–100 μ mol.L⁻¹ OTA, the NADPH-generating system containing 1 mmol.L⁻¹ NADP⁺, and 10 μ mol.L⁻¹ AAI dissolved in distilled water (see Materials and Methods).

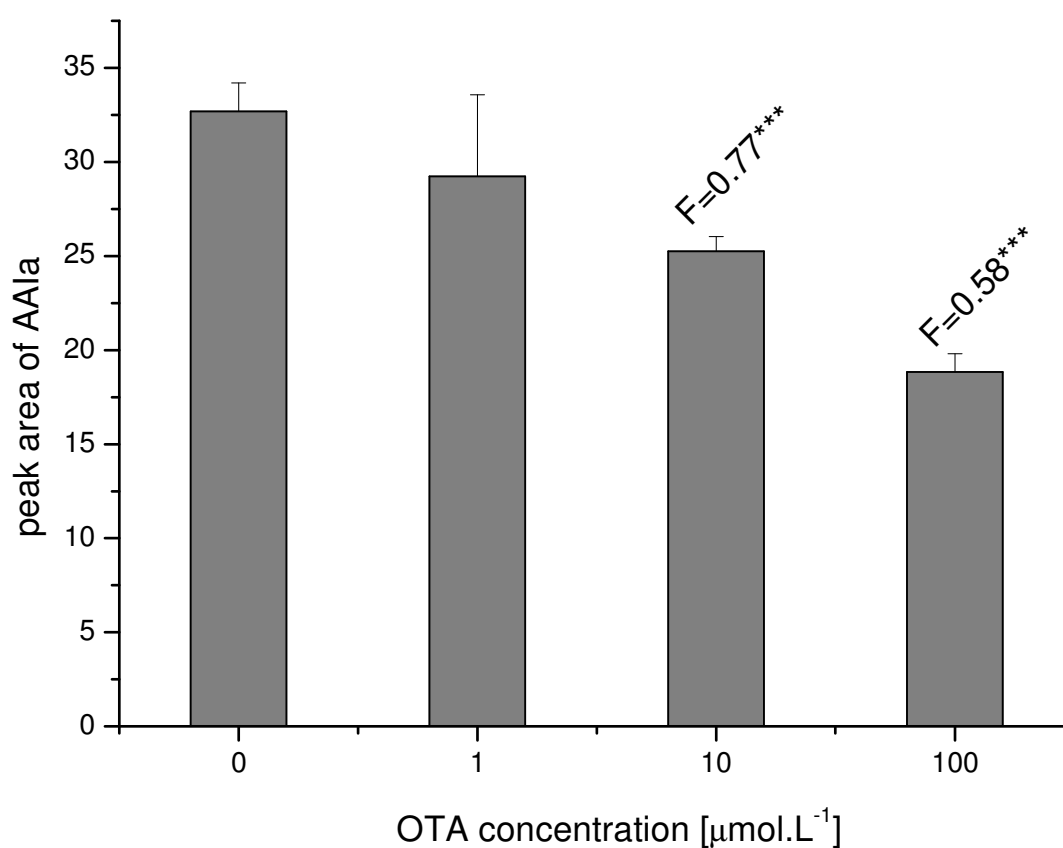


Fig. 6. Combined effect of cadmium and selenium ions, BBP and OTA on AAI detoxication to AAla. Incubations with CdCl_2 , Na_2SeO_3 and OTA (A) and in combination of these compounds with BBP (B) were carried out. Values are given as means \pm standard deviations of three experiments ($n = 3$). Values significantly different from incubation with buffer/acetonitrile only: * $P < 0.1$, ** $P < 0.01$, *** $P < 0.001$ (Student's t-test). The incubation mixtures contained 0.5 mg.mL^{-1} microsomal protein, $100 \text{ }\mu\text{mol.L}^{-1}$ CdCl_2 , Na_2SeO_3 , dissolved in distilled water, OTA dissolved in 0.1 mol.L^{-1} NaHCO_3 (pH 7) or BBP dissolved in acetonitrile, the NADPH-generating system containing 1 mmol.L^{-1} NADP^+ , and $10 \text{ }\mu\text{mol.L}^{-1}$ AAI dissolved in distilled water (see Materials and Methods). Numbers above the columns ("F") indicate fold changes in amounts of AAla compared to incubations without the above mentioned compounds.

